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Retinoids as Anti-Cancer Agents

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Abstract: Retinoids, synthetic and natural analogs of retinoic acid, exhibit potent growth inhibitory and cell differentiation activities which account for their beneficial effects in cancer in *ex vivo* and *in vivo* models. These simple molecules with pleiotropic effects have shown potential as therapeutic agents for the treatment of cancer either alone or in combination with other agents. Retinoids regulate the growth of various cell types by directly modulating the expression of responsive genes through nuclear retinoid receptors (RARs and RXRs), which are ligand dependent transcription factors. The translocation of RARα in acute pro-myelocytic leukemia, decreased expression of RARβ and reduced activity of the RARβ promoter in various tumors and cancer cell lines, and restoration of retinoid sensitivity to cancer cells by RAR expression vector transfection, are all indicative of the direct involvement of RAR malfunction in the process of tumorigenesis and also suggest a role for RARs as ligand dependent tumor suppressors. The current use of retinoids in cancer is limited because of their associated toxicities and lack of efficacy at tolerated doses. In order to improve the therapeutic index of retinoids, various strategies are currently being employed, e.g., receptor selective retinoids, anti-AP1 selective retinoids and combination therapies. The development of novel retinoids along with an increased understanding of the biological functions and mechanisms of action of retinoid receptors are likely to usher in a new era of retinoid therapy of cancers.

Cancers are a group of diseases which are characterized by hyperproliferation and uncontrolled growth of cells achieved by multiple pathways in an endocrine, paracrine or autocrine stimulus dependent manner. Thus, cancer cells may exhibit sustained growth in response to hormones (e.g., estrogen dependent breast cancer) and autocrine growth factors (e.g., IL-6, IL-8, VEGF, bFGF and oncostatin M in Kaposi's sarcoma). As the tumor grows, the endothelial cells in the vicinity are stimulated to form new blood vessels (neovascularization) supplying the tumor mass. The invagination and extension of newly formed blood vessels, the detachment of tumor cells from the primary site and attachment to new sites (mestastasis), are accompanied by turnover of the extracellular matrix (ECM), a process which requires a variety of metalloproteases capable of degrading ECM. Tumor cells themselves, or the surrounding tissue in response to stimuli from tumor cells may secrete the metalloproteases, which in turn initiate a series of localized proteolytic events necessary for cell motility and invasive phenomena through the tissue. This suggests that there are multiple ways of intervening in the process of carcinogenesis using small molecules [Fig. (1)]. Tumor growth can thus be stopped by interfering with the mitotic action of growth factors, either by repressing the transcription or translation of these factors or by interfering at various stages in their signal transduction pathways. Without angiogenesis, tumor cells may remain dormant (*in situ* carcinomas) for extended periods, since the expansion of *in situ* carcinoma into a malignant tumor requires the switching of tumor and /or surrounding endothelial cells into an angiogenic phenotype, recruitment of new blood capillaries, and the autocrine and paracrine stimulation of tumor cells by various growth factors. Therefore, angiogensis inhibitors should effectively inhibit the progression of neoplastic cells into malignant tumors and tumor metastases.

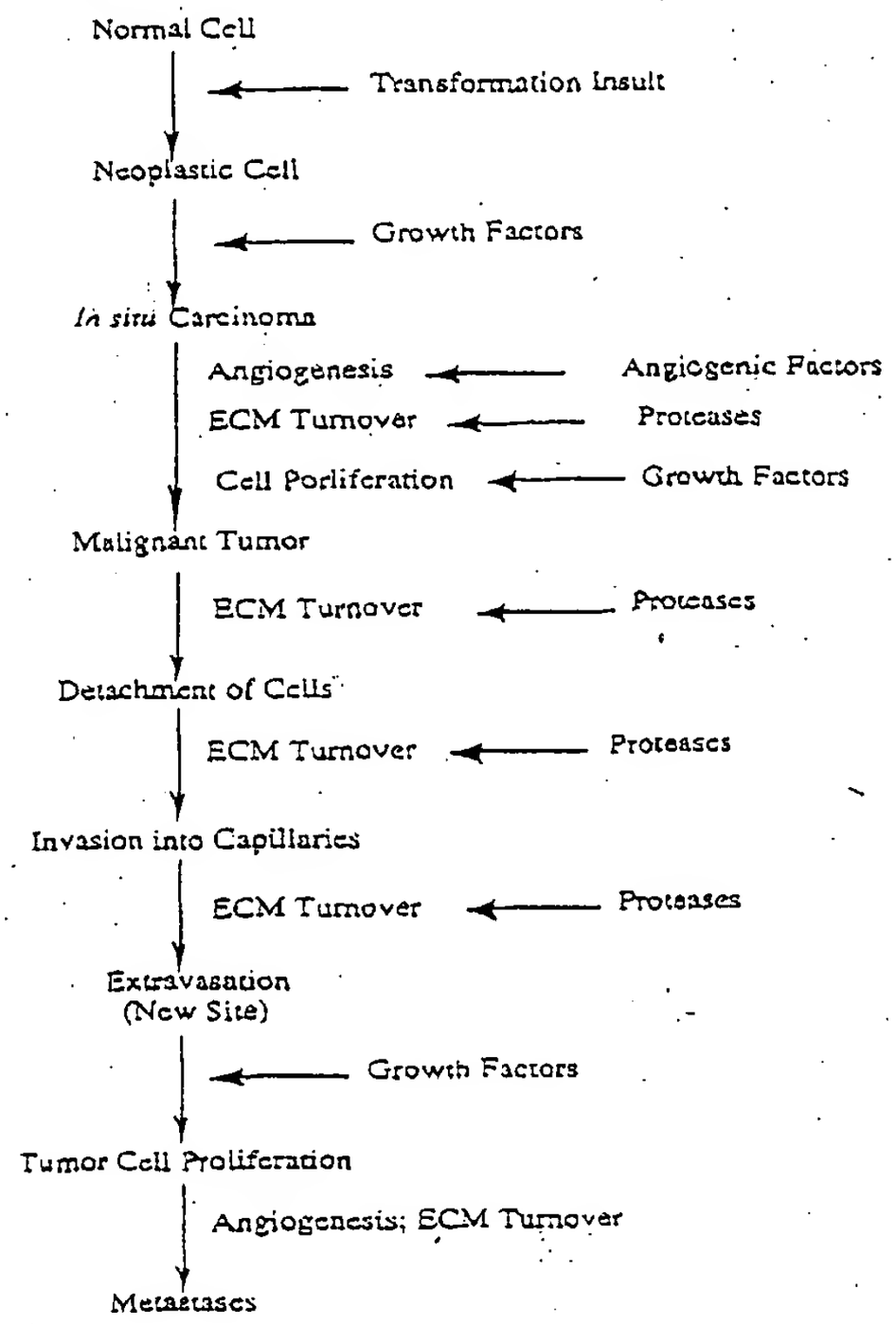

Fig. (1). The pathogenesis of tumor development and metastasis. For the development of a malignant tumor and metastases, the growth factor stimulated neoplastic cell mass must undergo a series of sequential steps involving angiogenesis, invasive phenotype and hyperproliferation. The enormous biochemical pathways involved in these steps provide multitude of opportunities to intervene in the process of tumorigenesis and metastasis using potent chemical entities.

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Positive Regulation of Gene Expression by RARs

RARs and RXRs induce transcription by binding to response elements (RAREs and RXREs) present in the promoters of RA-responsive genes. The list of RA-responsive genes is rapidly growing with the application of sophisticated techniques (subtraction hybridization and differential-display PCR) to the study of differential gene expression by retinoids. The retinoid responsive genes which contain RAREs are divided into 3 classes (Table II). Class I contains genes with RAREs arranged as a direct repeat spaced by 5 bases (DR-5), Class II contains genes with direct repeat elements spaced by 2 bases (DR-2) and class III encompasses the genes containing complex RAREs which do not fall into Class I or Class II (Table II). Genes with RXREs in their promoter elements are shown in Table III. The

Table II. RAR-Responsive Genes and Their RAREs.

Gene	RARE Sequence					Reference
Class I (DR-5)						
m RARβ2	GGTTCA	N5	AGTTCA			[33]
h RARβ2	GGTTCA	N5	AGTTCA			[34]
m RARα2	AGTTCA	N5	AGTTCA			[35]
h RARα2	GGGTCA	N5	AGGACA			[36]
h CRABP II	AGGTCA	N5	TGGGCA			[37]
h CMV-IE gene promoter	AGGGCA	N5	AGGTCA			[38]
r Neurogranin (RC3)	GGGTCA	N5	AGGTCA			[39]
h RARγ2	GGGTCA	N5	AGTTCA			[40]
h ADH3	AGGTGA	N5	AGGTCA			[41]
m Hox-4.2	GGTTCA	N5	AGTTCA			[42]
m Hox-1.6	GGGTCA	N5	GGGTCA			[43]
h t-PA	AGGTCA	N5	CGGGCA			[44]
h MK						
Class II (DR-2)						
m CRBPI	AGGTCA	N2	AGGTCA			[45]
m CRABP II (RARE II)	AGGTCA	N2	AGGTCA			[46]
m CRBP II (RE2)	AGGTCA	N2	AGGTCT			[47]
r CRBP I	AGGTCA	N2	AAGTCA			[48]
h APO AI	GGGTCA	N2	GGTTCA			[49]
Class III (Complex)						
m Laminin B1	AGGTGA	N3	AGGTTA	N14	GGGTCA	[50]
HIV-1 LTR	GGGTCA	N9	AGACCT			[51]
γF-Crystallin	TGACCC	N8	AGGTCA			[52]
h ICAM-1	GGGTCA	N0	TCGCCC			[53]
h Thrombomodulin	TGGTCA	N4	AGGTCA			[54]
h Medium chain Acyl CoA dehydrogenase	TGACCT	N8	GGGTAA	N0	AGGTGA	[55]
m Complement factor H	TCCAGG	N0	AGGTCA			[56]
h Osteocalcin	AGGTGA	N1	TCACCG			[57]
b Growth hormone	GGGACA	N0	TGACCC			[58]
r Growth hormone	GGGACG	N0	TGACGG			[59]

Prefixes in the lower case in front of the name of the genes are: m, mouse; h, human; r, rat and b, bovine. CRABP II is abbreviated for cellular retinoic acid binding protein II; CMV-IE, cytomegalovirus-immediate early; ADH3, alcohol dehydrogenase 3; t-PA, tissue plasminogen activator; MK, midkine; CRBPI, cellular retinol binding protein I; APOAI, apolipoprotein AI; HIV-1 LTR, human immunodeficiency virus-1 long terminal repeat; ICAM-1, intercellular adhesion molecule-1; "N" denotes the number of nucleotides present between each repeat motif.

direct repeat motifs are spaced by one base in RXREs (Table III), whereas they are generally spaced by 5 or 2 bases in the case of RAREs (Table II). Although a number of RAREs and RXREs are defined by transfection and *in vitro* gel retardation studies, their *vitro* and *ex vivo* systems used to characterize them.

Amongst the many genes regulated by retinoids in various systems, the induction of certain transcription factors is particularly interesting, since they in turn can modulate the

Table III. RXR-Responsive Genes and Their RXREs

Gene	RXRE Sequence									Reference
m CRABP II (RARE II)	AGGGCA	N1	AGGTCA							[46]
m CRBP II (RE1)	AGGTCA	N1	AGTTCA							[47]
m CRBP II (RE3)	GAGTCA	N1	AGGTCA							[47]
r CRBP II	CTGTCA	N1	AGGTCA	N1	AGGTCA	N1	AGGTCA	N1	AGTTCA	[59]
r PEPCK	CGGCAA	N1	AGGTCA							[60]
r Acyl CoA Oxidase	AGGACA	N1	AGGTCA							[61]
m MHC I	AGGTCA	N1	GGGTGG							[62]
h HBV	GGGTAA	N1	GGTTCA							[63]
h APO AI	AGGGCA	N1	GGGTCA							[49]

Various abbreviations used in the table are: PEPCK, phosphoenolpyrurate kinase; MHC I, major histocompatibility complex I; HBV, hepatitis B virus. The expanded versions of the rest of the genes are given in Table II legend. "N" denotes the number of nucleotides separating the two repeat motifs.

retinoid responsiveness *in vivo* remains to be confirmed. For example, mRARβ2 promoter is induced *in vivo* in developing mouse after RA treatment and a reporter under control of the mRARβ2 promoter is expressed at the sites where natural RARβ2 expression is observed [64]. In contrast, mCRBPII which contains both an RARE and an RXRE, was not induced in the intestine of RA or 9cis-RA treated mice. Furthermore, in an enterocyte-like cell line, CaCo-2, which consitutively expresses CRBPII, the transfected CRBPII promoter does not respond to either endogenous or overexpressed RARs and RXRs in the presence of RA or 9-cis-RA [47]. Similarly, an RARE has been identified in HIV-LTR by transfection and gel retardation based systems and HIV expression is both induced and inhibited by retinoids in a cell-context dependent manner in culture systems [65,66]. However, *in vivo* in HIV positive patients treated both topically and systemically with retinoids for Kaposi's sarcoma, viral load was not perturbed during therapy [67]. These results indicate that some genes may be erroneously identified as retinoid responsive because of the artifactual nature of the *in*

expression of genes required for differentiation or proliferation and trigger a cascade of biological effects. The transcription factors whose expression is induced by RA, are listed in Table IV.

Negative Regulation of Gene Expression by RARs

Genes whose expression is repressed by retinoids are listed in Table V. The known hyperproliferative and pro-inflammatory functions of several of these gene products suggest that many of the therapeutic effects of retinoids could result in part from their negative gene regulatory activities. RA inhibits the activity and/or expression of metaloproteinases (stromelysin-1, collagenase and 92kd gelatinase), oncogenes, growth factors and their receptors and ECM components (Table V), all of which are known to be involved in the processes of hyperproliferation, tumor development and metastasis. IL-6 is an autocrine growth factor for myeloma and Kaposi's sarcoma (KS) cells (our

Table IV. RAR-Reponsive Transcription factor genes

Transcription Factor Gene	Induction by RA *Ex Vivo*	Induction by RA *In Vivo*	Reference
RARβ2	+	+	[33, 34, 64]
RARα2	+	ND	[35]
RARγ2	+	ND	[39]
Zif 268/Krox-24/NGF1-A/Egr-1	+	+	[68-70]
GATA-4	+	ND	[71]
MZF-1	+	ND	[72]

All the genes encode zinc finger proteins, and with the exception of MZF-1, are proven transcription factors since they bind to specific DNA motifs and induce the expression of the promoters carrying those DNA sequences. MZF-1 is a putative transcription factor. ND- not determined.

Table V. Negative Regulation of Genes by RA

Gene	Negative Regulation by RA *Ex vivo* mRNA	*Ex vivo* Protein	Promoter Level	Reference
Proteases				
Stromelysin-1	+	+	+	[5,73,74]
Collagenase	+	+	+	[74-76]
Gelatinase	+	+	ND	[74]
Proto-oncogenes/ transcription factors				
c-Fos	+	+	+	[77, 78]
N-Myc	+	ND	+	[79]
c-Myc	+	+	+	[80, 81]
ODC	+	+	+	[82]
HPV-E6, E7	+	+	+	[83, 84]
Oct - 3/4	+	+	+	[85, 86]
Growth factors and their receptors				
TGF-β1	ND	ND	+	[87]
EGF-R	+	+	ND	[88]
IL-6	+	+	+	[89]
IL-6R	+	ND	ND	[90]
Cell adhesion molecules and structural proteins				
β4-integrin	+	+	ND	[91]
Fibronectin	+	+	ND	[92]
K5	+	+	+	[93, 94]
K6	+	+	+	[93, 95]
K14	+	+	+	[93, 94]
K16	+	+	ND	[93]
Loricrin	+	+	+	[96, 97]
SKALP	+	+	+	[89]
Enzymes				
CD 13	+	+	ND	[98]
Carbonic anhydrase	+	+	ND	[99]
Protein phosphatase 2A	+	+	ND	[100]
Tyrosinase	+	+	ND	[101]
TGase I	+	+	+	[102, 103]
GST-P1-1	+	+	+	[104]
Pro-inflammatory proteins				
JE/MCP-1	ND	+	ND	[105]
iNOS	+	+	ND	[106]
TNF-α	+	ND	ND	[107]
IL-2	ND	+	+	[107a]
MRP-8	+	+	+	Our unpublished results
Others				
CT/CGRP	+	ND	+	[108]
Oxytocin	ND	ND	+	[109]
IGFBP-2 and 4	ND	+	ND	[101]

The table depicts the genes which are repressed after RA treatment in various culture systems. The repression of protein content/activity, mRNA level or promoter activity in transiently transfected cells, of a given gene by RA, which is documented in literature, is presented. The abbreviations used in the table are: ODC, ornithine decarboxylase; HPV-E6, E7, Human papilloma virus 16/18-E6 and E7 oncoproteins; TGF-β1, transforming growth factor-β1; EGF-R, epidermal growth factor receptor; IL-6, interleukin-6; IL-6R, IL-6 receptor; TGF-α, transforming growth factor-α; K5, keratin 5; GST-P1-1, glutathione-S-transferase-P1-1; MCP-1, monocyte chemotactic protein-1; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α; MRP-8, macrophage migration inhibitory factor related protein-8; CT/CGRP, calcitonin/calcitonin gene related peptide; IGFBP, insulin like growth factor binding protein. ND-Not determined.

unpublished results) and is down-regulated by retinoids. A number of these genes are negatively regulated by RA at the promoter level in transient transfections (Table V). The negative regulation by retinoids of protein and/or mRNA levels of single copy, endogenous genes has also been demonstrated in several cases in *ex vivo* conditions [73,5]. Also, many of these negatively regulated gene products are regulated in the same direction in various disease situations where retinoids are known to exert their therapeutic benefits. Some examples of such negative regulation are as follows: the expression of stromelysin-1, collagenase and 92kd gelatinase is repressed by RA in UV-treated skin [74]; hyperproliferation associated keratins K6 and K16 are down-regulated in psoriatic lesions after treatment with tazarotene, an RAR-selective synthetic anti-psoriatic retinoid [110 and our unpublished results]; ODC activity is repressed after treatment of psoriatic lesions with a synthetic retinoid, etretinate [111]. The shift in emphasis in retinoid research from phenomenology to the molecular basis of action will lead to the identification of many more retinoid regulated genes and a better understanding of which regulations are important for retinoid efficacy and toxicity. These precise molecular targets will enable the design of more specific retinoids with improved therapeutic indices and suggest new approaches to the treatment of retinoid responsive diseases including cancer.

RAR-RXR Heterodimers Transduce Retinoid Signals

RARs are believed to function exclusively *in vivo* as RAR-RXR heterodimers. Based on the observation that co-factors present in nuclear extracts are required for high-affinity binding

of RAR, VDR and TR to their cognate responsive elements [112-114], a number of groups using different approaches simultaneously demonstrated that the heterodimeric partner for RARs is RXR [24,61,115-118]. RAR heterodimerizes with RXR in solution and this RAR-RXR heterodimer binds with a high affinity to an RARE *in vitro*. The next obvious question was, whether RAR-RXR heterodimers or RAR homodimers induced gene expression inside a cell. To answer this the RARE present in the CRBPII promoter was replaced with a GAL4 DNA binding site, thus making the CRBPII promoter unresponsive to transfected RAR or RXR. A reporter gene with this modified promoter did not respond to Gal4 RXR(DE) expression vector in the presence of 50 nM RA in transfected cells but responded to co-transfection with GAL4 RXR(DE) and RAR expression vectors, thus demonstrating that the RAR-RXR heterodimer is transcriptionally functional inside the cell. Similar results were obtained by Gal4 RAR(DEF) and RXR co-transfections in the presence of ligand and by Gal 4 RXR(DE) and VP16-RARγ in the absence or presence of ligand [20]. Further, by using VP16-RAR, the possibility of RAR or RXR homodimer formation inside the transfected cells was ruled out [20]. Subsequently, it was found that RXR always occupies the 5' site of the direct repeat motif [119] and on a DR-5 or DR-2 motif, only the RAR LBD but not the RXR LBD is occupied by the ligand [120].

RXR as a Heterodimeric Partner for Other Nuclear Receptors

Interestingly RXRs can also form heterodimers with Vitamin D receptor (VDR), thyroid hormone receptor (TR), peroxisome-proliferator activated receptor (PPAR), COUP and ARP-1 [61,115-122]. All of these heterodimers bind to response elements arranged as direct repeats (DR) separated by varying number of nucleotides. RXR-PPAR heterodimers, in addition to RXR homodimers, bind to DR-1 (RXRE or PPRE , direct repeat of 5'-PuGG/TTCA-3' repeat with 1 base pair spacing) and transactivate promoter-reporter constructs containing DR-1 motifs. The DR-2 and DR-5 motifs are RAREs since they have affinity for RAR-RXR heterodimers. The DR-3 motif is a vitamin D responsive element (VDRE), since it binds and transcriptionally responds to VDR-RXR heterodimers. Similarly, the DR-4 motif is a TRE (thyroid hormone responsive element) since it is a high affinity binding and transcriptionally active motif for TR-RXR heterodimers. This 1-2-3-4-5 rule dictates the binding of various heterodimers to their cognate responsive elements and is the molecular basis for the target gene specificity observed for the various nuclear receptor hormones [46,119,121,123]. The hererodimerization of RXRs with other nuclear receptors enhances their DNA binding and ligand-dependent transactivation capability through their specific response elements. The exceptions in the paradigm of transcriptional activation by heterodimers are COUP-RXR and ARP-1-RXR heterodimers, which bind co-operatively to the DR-1 response elements but act as potent repressors of RXR-RXR homodimer mediated transcriptional activation [61,120]. COUP and ARP-1 are orphan receptors whose ligands have not yet been identified. The DR-1 binding PPAR was recently shown to bind and be activated by the naturally occurring prostaglandin, 15-deoxy- Δ12, 16-prostaglandin J2 (15d PGJ2) [124,125]. PPAR is also activated by hypolipidemic drugs, such as chlofibric acid, fatty acids and the anti-diabetic thiazolidinediones. PPREs (PPAR-responsive elements) have been identified in the promoter sequences of rat acyl-CoA-oxidase, 3-ketoacyl-CoA-thiolase, chicken ovalbumin and CRBPII promoters. PPARα-RXRα heterodimers transactivate rat acyl CoA-oxidase PPRE in response to both 9cis-RA and clofibric acid and a simultaneous exposure to both ligands results in synergistic activation [61]. In contrast, PPARγ-RXRα heterodimers transactivate rat acyl-CoA oxidase PPRE only in the presence of 15d PGJ2 but not in the presence of clofibrate, WY14,643 (a clofibrate analog) or LG69 (an RXR-specific ligand) [124].

VDR-RXR heterodimers bind and transactivate through a DR-3 motif, unlike VDR homodimers which selectively induce the expression of a DR-6 reporter motif construct [126]. 1,25-dihydroxyvitamin D_3, the physiological ligand for VDR, favors the formation of VDR-RXR heterodimers over VDR homodimers. Synergistic action between 1,25-dihydroxyvitamin D_3 and 9cis-RA has been observed for the transcriptional activation of synthetic DR-3 and osteopontin promoters [126]. The response elements for TR-RXR heterodimers are characterized by DR-4 motifs. Both thyroid hormone (T3) and 9cis-RA activate the expression of a TRE reporter in transfected cells, and a combination of the two ligands synergistically induces the expression of growth hormone TRE in JEG-3 cells [127]. Although synergistic interactions between RXR ligands and RAR, VDR, TR or PPAR agonists have been reported in the literature in a variety of transfected and non-transfected cells, such activities have not yet been reported in *in vivo* models.

Positive and Negative Co-factors of RAR Action

Studies with steroid hormone receptors have shown that the estrogen receptor (ER) can interfere with the transcriptional activity of the progesterone receptor (PR) or glucocorticoid receptor (GR) on PR or GR promoter elements which do not bind to ER [128]. Similarly, high level expression of Gal4 or Gal4-VP16 interfered with transcription from genes lacking the Gal4 binding motifs [129,130]. The above squelching studies along with the findings regarding the cell and promoter-context dependency of activation functions (AF-1 and AF-2) of nuclear receptors [20,22,131], suggested the existence of co-activators which interact with the nuclear receptors. Thus, transactivators, including the steroid hormone receptors, function by interacting with intermediary co-factors, which are present in limiting amounts in the cell. These factors may be widely shared between the transcriptional activators, as in the case of co-factors required for ER, PR, GR and Gal4. Evidence for the presence of RAR AF-2 co-factor activity has come from studies in lung cancer cell lines which display low or negligible levels of RARβ2 mRNA expression. Nuclear run-on assays and transient transfection of RARβ2 promoter constructs indicated the presence of a general block of transcriptional activity in these cells. Further studies indicated that this transcriptional block in lung cancer cells derived from a deficiency of specific co-factor activities required for RAR function [132].

Another set of elegant experiments utilizing Gal4 chimaera suggested for the first time the existence of negative co-factors or co-repressors which interact with nuclear receptors [133]. In these experiments, Gal4-VP16 activated Gal4 responsive reporter in the absence or presence of T3, whereas, Gal4-TR

(containing Gal4 DBD and TR LBD) activated Gal4 reporter only in the presence of thyroid hormone (T3). The transactivation capability of Gal4-VP16 was abolished by insertion of the TR C-terminal region into Gal4-VP16 (Gal4-TR-VP16) and co-transfection with TR, RAR or RXR restored the transcriptional activity of Gal4-TR-VP16. These results suggested the presence of a co-repressor protein which interacted with the TR C-terminal region of Gal4-TR and Gal4-TR-VP16 and suppressed the ability of these chimaeric proteins to transactivate from the Gal4-reporter. Treatment with T3 resulted in transactivation through Gal4-TR because interaction with co-repressor was destabilized in the presence of hormone. Similarly, co-transfection with TR, RAR or RXR resulted in de-repression of Gal4-TR-VP16 activity because the putative co-repressor was sequestered away from Gal4-TR-VP16 by the transfected nuclear receptor.

More recently, using yeast two hybrid systems, a number of laboratories have identified a variety of positive co-factors or transcriptional intermediately factors (TIFs) which interact with the nuclear receptors in a ligand dependent manner. These include a mouse bromodomain-containing protein, TIF1 [133a], the ER-associated proteins ERAP 160 [134], RIP160 and RIP180 [135], the thyroid hormone receptor interacting proteins (TRIPS) [136, 137], SUG1 (TRIP1) [136, 138] and SRC-1 [139] (Table VI). Most of these proteins interact with the AF-2 regions of multiple nuclear receptors and thus explain the initial observations of squelching or transcriptional interference between the activation functions of various transcription factors. SUG1 interacts with the AF-2 domain of ER,TR,VDR, RXR and RAR [138]. Likewise, SRC-1, isolated as a PR interacting protein, exhibited ligand dependent interaction additionally with GR, ER, TR and RXR [139]. As expected of positive co-activators, the interaction of these proteins with the receptor is neither observed in the absence of agonist, nor in the presence of an ER antagonist (hydroxytamoxifen) or a PR antagonist (RU-486) for ER or PR interacting proteins [133, 139]. Additionally, SRC-1 has been shown to be a functional co-activator since it enhances receptor-mediated transcription activity [139].

Table VI. Co-activators and co-repressors of nuclear receptors

Factor	Receptor	Reference
Co-activators (TIFs)		
TIF1	ER,RAR,VDR, RXR, TR	[133a,136,138]
TRIP1	TR	[136, 137]
SUG1	RAR, ER, TR, VDR, RXR	[138]
ERAP 160	ER, RAR, RXR	[134]
RIP 160	ER	[135]
RIP 180	ER	[135]
SRC-1	PR, GR, ER, TR, RXR	[139]
Co-repressors		
N-CoR	TR, RAR	[21]
SMRT	TR, RAR, RXR	[140]

Various co-activators and co-repressors along with the nuclear receptors with which they have been documented to interact are presented.

Also, using a similar yeast two hybrid screening strategy, two groups have very recently isolated two distinct co-repressors of RAR action (Table V). These co-repressors interact with the receptor in the absence of ligand and ligand binding destabilizes RAR/co-repressor interaction. One of these co-repressors, N-CoR, interacts with the hinge region (region D) of both RAR and TR, while the other, SMRT (silencing mediator for retinoid and thyroid-hormone receptors), interacts with the LBD of RAR, TR and RXR in the absence of agonist. Both N-CoR and SMRT are true transcriptional co-repressors, since they can transfer active repression to a heterologous DNA binding domain [21,140].

Cross-Talk of RARs With Other Signal Transduction Pathways

Retinoids exert both positive and negative gene regulatory effects. The positive gene regulatory effects can be explained by the ability of RAR-RXR heterodimers to transactivate expression of target genes which have RAREs in their promoter regions. The genes which are negatively regulated by RARs (Table V), do not appear to have any RAREs in their promoter regions. The exception is the oxytocin gene which has a negative RARE in its promoter [109]. This novel mode of negative regulation, without DNA binding, is achieved by antagonism of the enhancer activity of certain other transcription factors by either direct protein: protein interaction between RARs and these transcription factors or indirect interaction involving an intermediary protein or co-factor.

RAR-AP1 Antagonism

AP1 and RARs are effectors of opposite signal transduction pathways. AP1, a complex of the oncogene products c-Jun and c-Fos, is involved in cell proliferation while RARs have anti-proliferative and cell differentiation effects. AP1 and RARs mutually antagonize each other at the level of transactivation [5,57,73,141]. The antagonism results in RA-mediated inhibition of expression of certain genes which solely depend upon an AP1 motif for their transcription (Table VII). Unlike RAR-mediated transactivation which involves protein-DNA interaction between RAR-RXR heterodimer and RAREs, RAR-mediated antagonism of AP1-dependent gene expression does not involve binding of RARs or RAR-RXR heterodimers to the AP1 motif [73]. Thus, RARs may antagonize AP1 function by binding directly to c-Jun/c-Fos to form an inactive complex or by interacting with and sequestering other nuclear accessory

Table VII. Inhibition of AP1-dependent gene expression by RA

Gene	AP1 Sequence	Reference
Stromelysin-1	TGAGTCA	[5, 73]
Collagenase	TGAGTCA	[5,7,141]
TGF-β1	TGACACG TGAGACT	[87]
HPV-18 regulatory region	TTAGTCA TGACTAA	[84]
TGase I	TGACTCA TGAGTCA	[103]
Glutathione-S-transferase	TGACTACA	[104]
K6	TGACTAA	[95]
Loricrin	TGACTAA TGAGTCA	[96, 97]

Genes which contain at least one AP1-motif in their upstream sequences and whose expression is repressed in a retinoid dependent manner are listed here in. The sequence of their AP1 motif is also presented.

factors that are required for AP1-mediated transcriptional activation [5]. Recently, CBP (CREB-binding protein), a co-factor required for CREB and c-Jun mediated transactivation was found to interact with RAR, indicating that ligand dependent RAR-CBP interaction is a plausible mechanism for retinoid-mediated anti-AP1 activity [142 and our unpublished results].

The involvement of the AP1 motif in retinoid-dependent negative regulation of collagenase, stromelysin-1, TGF-β1, and HPV-18 regulatory regions has been documented [5,57,73,84,87,141]. The elevation of metalloproteinases can engender an invasive phenotype to tumor cells and facilitates tumor metastasis [143]. The growth factor TGF β1 is produced by many tumor cells [144] and the HPV-18 regulatory region controls the transcription of transforming oncoproteins E6 and E7 [84]. Thus, the cross-talk between RAR and AP1 signal transduction pathways could be therapeutically manipulated for the treatment of various hyperproliferative conditions by antagonism of AP1-regulated gene transcription. However, the wider use of retinoids is often hampered by the toxicities associated with retinoid therapy [145]. In order to improve therapeutic indices of future retinoids, attempts have been made to produce transcriptionally inert synthetic retinoids with only AP1-antagonism properties, and recent studies have suggested that this pharmacological separation of transactivation and AP1 antagonism functions of RARs may be possible [5,146]. As to what complement of therapeutic and toxic effects of retinoids are associated with the AP1 antagonism or transcriptional activation properties remains unknown and could effectively be determined only by the development of analogs that are specific for either the positive or negative gene regulatory pathways.

RAR-NF-IL6 Antagonism

AIDS-Kaposi's sarcoma (KS) cells in culture are responsive to a variety of growth factors including IL-1, IL-6 and oncostastin M, which are autocrine growth factors for KS cells [147-151]. Retinoids inhibit the proliferation of KS cells in culture and RA has been of benefit in treating KS lesions in AIDS-KS patients [67,152]. It has recently been discovered that RA inhibits the expression of IL-6 in KS cells in culture. The expression of an IL-6 promoter reporter construct is also inhibited by RA and the element responsible for RA-mediated inhibition of IL-6 gene is the NF-IL6 motif since the expression of a reporter gene containing an NF-IL6 motif, in the context of a herpes simplex virus truncated thymidine kinase gene, was also repressed by RA in transfection assays [our unpublished results]. Thus, in addition to AP1, NF-IL6 is another bZip (basic leucine zipper) transcription factor whose enhancer action is antagonized by RA.

RAR-BZLF1 Antagonism

Epstein-Barr Virus (EBV), a human herpes virus, is associated with B-cell lymphomas and nasopharyngeal and parotid carcinomas. The infection of B-cells by EBV is predominantly latent, whereas infection of epithelial cells usually leads to a lytic cycle of viral replication. The switch from viral latency to productive infection occurs through a number of agents which mediate their effects through the EBV immediate early gene product, BZLF-1 (also referred to as protein Z, ZEBRA, ZTa or EB-1). Overexpression of BZLF1 is sufficient to disrupt viral latency and trigger viral replication. BZLF1, like c-Jun/c-Fos and NF-IL6, is a member of the basic leucine zipper (bZIP) family and it transactivates the viral early genes which contain AP1 like motifs in their promoters [153,154]. Since BZLF1 is the major player in the disruption of viral latency, it could be a target for drugs that prevent the virus from entering into a productive lytic cycle. The cross-talk between RARs and bZIP family members extends to BZLF1 as well and RARs might well be the cellular factors which maintain the virus in a latent state. RAR and RXR both exhibit direct protein: protein interaction with BZLF1 and the DBD of RARα and the transactivation and homodimerization domains of BZLF1 are required for this interaction [153;154]. Therefore, BZLF1 is a new target of retinoid action which may potentially be exploited for the treatment of EBV-induced malignancies.

RAR-ER Interaction

Cross-talk between the retinoid and estradiol (E_2) signal transduction pathways is suggested by the observations that RA selectively inhibits the proliferation of ER-positive human breast cancer cells, while ER-negative cancer cells are refractory to the growth inhibitory effects of RA [155-158]. Further, the

ER-positive breast cancer cells exhibit increased sensitivity to the growth inhibitory action of RA in the presence of E_2 [159]. A possible reason is that E_2 increases RARα gene expression in ER-positive breast cancer cells via an imperfect half-palindromic estrogen responsive element (ERE) and a Sp1 motif present in the promoter region of RARα1 [159,160]. Accordingly, ER-negative breast cancer cells transfected with ER expression vector exhibit increased RARα gene expression and responsiveness to growth inhibition by RA [161]. In addition to potently inhibiting the proliferation of hormone-dependent breast carcinoma cells *ex vivo*, retinoids synergize with ovariectomy and anti-estrogens in growth inhibition of breast tumors *in vivo* [162-164]. In a simple model of RAR-ER cross-talk, E_2 induced the expression and synthesis of growth factors which resulted in uncontrolled proliferation of the cells and retinoids antagonized the E_2-dependent gene expression, thereby repressing growth factor activities. Several studies have in fact indicated that retinoids have properties that are functionally equivalent to that of anti-estrogens [165-167]. Apart from inducing gene expression from an ERE, ER can also induce the activity of certain promoters by interacting with Sp1 and by inducing AP1 (Jun-Fos) activity. ER-Sp1 interaction activates the c-myc promoter [168] and E_2 activates the insulin like growth factor 1 (IGF 1) gene by increasing AP1 activity [169]. E_2 also increases the secretion of platelet derived growth factor (PDGF) and transforming growth factor-α (TGF-α) in ER-positive breast cancer cells [170-172]. It should be noted that TGF-α expression is inhibited by RA in squamous cell carcinoma (SCC) cells [173]. The antagonism of ER-mediated gene expression by retinoids can possibly be explained at the molecular level on the basis that ER and RAR share common co-activator proteins (TIF1, SUG1, ERAP 160), which are required for the AF-2 functions of both classes of receptors [133a,134,138].

RARs as Tumor Supressors

The inactivation of tumor suppressor genes by mutation or deletion releases the cells from the growth inhibitory constraints imposed by these genes, thereby resulting in the uncontrolled growth of cancer cells and the genesis of a variety of human tumors [174, 175]. Some of the known tumor suppressor genes are DCC (deleted colon carcinoma), NF-1 (neurofibramatosis-1), Rb (retinoblastoma), WT (Wilm's tumor) and p53 [175]. The following lines of evidence strongly suggest that RARs may also be classified as tumor supressors: (a) Inactivation of RARα by translocation in acute promyelocytic leukaemia (APL), (b) loss of RARβ expression in human lung cancer, premalignant oral lesions, oral SCC cells and renal cell carcinoma, (c) loss of RARα expression in retinoid resistant breast cancer cells, (d) restoration of retinoid responsiveness by RAR transfection in previously resistant cells, (e) inhibition of oncogene-induced focus formation by RARβ2 and (f) RAR-dependent inhibition of expression and/or activities of certain transforming oncogenes.

Inactivation of the RARα Gene in APL

APL is a rare form of acute myeloid leukaemia (AML), which is characterized by a reciprocal chromosomal translocation (15;17) (q22; q11-22), and it is very responsive to RA therapy [176]. The localization of the human RARα gene to chromosome 17q led to the speculative link between the etiology of APL and the disruption of RARα gene by translocation [177]. Subsequently, a number of laboratories reported that the chromosomal 17 breakpoint in fact occurs in the RARα gene and the chromosome 15 breakpoint lies within a novel gene, PML or myl [178-182]. This reciprocal translocation results in the expression of aberrant PML-RAR (myl-RAR) and RAR-PML (RAR-myl) fusion transcripts [179,182,183-186]. PML-RAR exhibits altered transactivation properties as compared to wild type RARα and instead of inhibiting AP1-dependent gene expression, PML-RAR is a RA-dependent activator of AP1 activity [182,186-188]. PML-RARα protein heterodimerized both with PML and RXR and APL cells display an abnormal subcellular localization of PML-RARα and RXR [182]. Overexpression of PML-RARα fusion protein expression inhibits the differentiation of myeloid precursor cells U937 in response to stimuli (vitamin D_3 and TGF β1) and results in increased growth rate as a consequence of reduced apoptotic cell death [189]. In summary, a genetic lesion that interferes with the normal activity of RARα appears to contribute to the pathogenesis of APL, a situation which is analogous to the disruption of classical tumor suppressor genes in various human cancers. APL is very effectively treated with RA which restores a normal pattern of distribution of PML-RARα and RXR and presumably restores normal RARα function [190].

Abnormalities of RARβ Expression in Premalignant Lesions and Cancer Cells

RA deficiency is associated with squamous metaplasia in animals and a higher incidence of epidermoid (squamous) lung cancer in humans [for references see 191]. Further, in lung cancer cells, particularly in epidermoid lung cancers, non-random heterozygous loss of the short arm of chromosome 3(3p), where the RARβ gene resides, has been observed [192]. These observations led several laboratories to examine RARβ expression in various tumor cell lines. RARβ is expressed in normal lung tissue, and cells lines derived from adenocarcinoma and large cell carcinoma of lung, but is either reduced or absent in most of the cell lines derived from epidermoid or small cell lung carcinoma [191,193]. The reduced or complete absence of RARβ pertains primarily to RARβ2, since it is the most abundant RAR isoform in normal lung tissue [132,194]. Approximately 50% of the primary lung tumor are RARβ negative [32]. Further, RA does not induce RARβ in a third of the lung cancer cell lines, thus indicating a defect in the RA signal transduction pathway [193]. RARβ2 contains a DR-5 RARE in its promoter (table II) but the RARβ2 promoter is not activated in response to RA in lung cancer cell lines, whereas, a transfected RARβ2 DR-5 element in the context of a herpes simplex virus thymidine kinase promoter is active. This demonstrates promoter context dependent abrogation of retinoid responsiveness in lung cancer cells. These results also explain the reduced or complete absence of expression of RARβ2 gene in lung cancer cells [195]. The loss of RARβ2 expression has been attributed to inactivation of RAR AF-2 specific co-activators [132]. Similarly, RARβ expression is suppressed in many head and neck squamous cell carcinoma (HNSCC) [196] and renal cell carcinoma cell lines [197]. An RARβ2 promoter reporter construct in the context of its own promoter is not activated by

either RA or 9cis-RA in HNSCC cells, while a synthetic RARE reporter responds to RA and 9cis-RA in a dose dependent manner [196]. These results also demonstrate that although retinoid responses are selectively diminished in these cells, they are not entirely abrogated [198]. Finally, RARβ2 expression is inhibited in oral premalignant lesions and it is restored in the lesions of patients who show clinical responses to isotretinoin (13cis-RA) [199].

Restoration of Retinoid-Responsiveness in RAR-Transfected Cells

Transfection of tumor suppressor genes p53 and Rb can confer density dependent growth inhibition of tumor cells and block the progression of cells from G_0/G_1 to S phase of the cell cycle [200, 201]. In an analogous manner, RARα transfection into ER-negative, retinoid resistant, breast cancer cells results in retinoid dependent inhibition of proliferation of the transfected cells [202]. Further, transduction of RARβ cDNA into RARβ-negative breast cancer cells, MCF-7 and MDA-MB-231, also results in an RA-mediated inhibition of proliferation and induction of apoptosis in these cells [203]. Moreover, RARβ-transfected epidermoid lung cancer cells are markedly less tumorigenic in nude mice as compared to the untransfected cells which do not show RARβ2 expression. Also, the tumors which are produced from RARβ-transfected cells display reduced growth rate and increased tumor latency [191].

Inhibition of Oncogene Mediated Transformation by RARs

Wild type p53 inhibits the transformation and oncogene induced foci formation in fibroblasts transfected with the oncogenes Ha-Ras and p53 val 135 [204, 205]. The number of foci in Ha-Ras and p53 val 135 transfected fibroblasts were reduced by 65% by co-transfection with wild-type p53 and 76% by RARβ2 [206]. Another example of retinoid inhibition of oncogene-mediated transformation was observed in the JB-6, promotion sensitive (P+) mouse epidermal cell system. The tumor promoter TPA (12-O-tetradecanoylphorbol-13-acetate) and epidermal growth factor (EGF) induce the irreversible formation of large, tumorigenic, anchorage-independent colonies of JB-6, P+ cells in soft agar at high frequency. Both TPA and EGF are known to induce c-Jun expression and transactivation through AP1-motifs [for references, see 73]. RA blocks the TPA induced transformation of JB6 P+ cells in a concentration dependent manner [207].

RAR-Dependent Inhibition of Expression and/or Activity of Oncogenes

Interference with oncogene signal transduction pathways is a major mode of action of anti-oncogenes. NF-1 may act as a frank down-regulator of p21 ras and thus block ras-mediated mitogenic signaling. Cells transfected with Rb cDNA show decreased c-fos expression and Rb mediates TGF-β1-induced down-regulation of c-myc expression [for references, see 175] in keratinocytes. Similar inhibition of oncogene expression or antagonism of oncogene-mediated signal transduction pathway is being increasingly recognized as the basis of therapeutic action of retinoids in hyperproliferative diseases. Retinoids inhibit the

expression of c-fos, N-myc, c-myc, ODC and HPV-E6, E7 oncoproteins (Table V). ODC, the key regulator of polyamine biosynthesis is an oncogene since it is critical for cell proliferation and transformation [208] and ODC expression is effectively inhibited by retinoids. Further, RARs inhibit the expression of a number of genes which are controlled by oncogene products c-Jun and c-Fos (AP1) (Table VII).

Inhibition of Angiogenesis by Retinoids

The growth of solid tumors and their metastases requires the formation of a blood capillary network traversing the cellular mass of the tumor. This process of neovascularization or angiogenesis involves de-differentiation of the endothelial cells present in the vicinity of the tumor, then migration, invagination and proliferation, and finally their differentiation into quiescent endothelial cells forming the vascular structure. Since, retinoids are modulators of differentiation and they inhibit proliferation of endothelial cells in a dose-and time-dependent manner [209], various groups have analyzed the anti-angiogenic properties of natural and synthetic retinoids. The anti-angiogenic potentials of RA, retinyl acetate, a synthetic retinoid (Ch55) and a known inhibitor of angiogenesis, herbimycin A, were compared in a chicken embryo chorioallantoic membrane (CAM) assay [210]. These retinoids were strong inhibitors of embryonic angiogenesis and their rank order potency was Ch55>RA>herbimycin A> retinyl acetate. In a continuation of this study, three additional synthetic retinoids (Re 80, Am 580 and Am 80) were assessed in the CAM assay. Re 80, Am 580 and Am 80 were also effective inhibitors of angiogenesis in this *in vivo* model with ID_{50} (dose required for 50% inhibition of capillary formation) values of 6.3, 23 and 28 pmol/egg, respectively [211]. The ID_{50} values for inhibition of angiogensis by RA and Ch55 were 330 and 22 pmol/egg, respectively [210]. It is interesting that all synthetic retinoids tested were more potent than herbimycin A in this assay. RA, 9cis-RA and 13cis-RA have also been shown to inhibit angiogenesis in an *in vivo* tumor-induced angiogenesis (TIA) model. In the TIA model, these retinoids exhibited more than additive inhibition of angiogenesis, when used in combination with interferon α (IFN-α) and VDR agonist 1,25-dihydroxyvitamin D_3 [212, 213]. Further investigations into the biology underlying the anti-angiogenic effects of retinoids are required to determine whether this is a therapeutically useful effect of retinoids. With a more detailed understanding of this biology, it may be possible to develop newer subtype and function selective retinoids which might have useful anti-cancer effects by anti-angiogenic mechanisms.

Retinoids and Cancers

Squamous Cell Carcinoma (SCC) of Skin

SCC of skin is the second most prevalent cutaneous malignancy with basal cell carcinoma, (BCC) being the most prevalent. SCC occurs mainly on the sun-exposed areas of the body. It is a malignant tumor of keratinocytes which originates from a dysplastic epithelium and presents as islands of over-differentiated (keratinized) squamous epithelia in the epidermis and dermis. Oral isotretinoin (13cis-RA) has been successfully

used (50% response) for the treatment of advanced SCC of skin [214]. A number of patients in this study showed complete remission. Since combinations of retinoids and IFN-α had shown synergistic anti-proliferative effects in HL-60, U937, primary leukaemic, neuroblastoma, SCC and MCF-7 cells [for references, see 215], a clinical study was conducted with a combination of 13cis-RA and IFN-α in patients with advanced SCC. The overall response rate was approximately 70% and the complete response rate was 25%. Lower response rates were observed in patients with advanced metastatic tumors. Toxicities associated with IFN-α (fatigue) and 13cis-RA (mucocutaneous toxicity) were observed [216].

Head and Neck SCC (HNSCC)

HNSCCs, which account for approximately 4% of all human cancers, occur mostly in the oral cavity, larynx and pharynx [217]. The premalignant lesions are characterized by epithelial hyperplasia or dysplasia and include leukoplakia and erythroplakia. HNSCCs range from poorly differentiated to well differentiated tumors. Most of the oral cavity epithelium is non-keratinizing, but it can undergo keratinization in cancers (SCCs) or in vitamin A deficiency. Several clinical trials have suggested that β-carotene and retinoids reverse premalignant oral leukoplakia lesions [218, 219]. Oral 13cis-RA, with or without IFN-α was used for the treatment of advanced HNSCC but response rates were poor [220]. However, 13cis-RA is effective in preventing second primary tumors in patients who have been treated for HNSCC, although it does not affect the recurrence of the original tumor [221]. Theoretically retinoids offer significant potential in the treatment of HNSCC, since they alter the expression of oncogenes, growth factors and their receptors, whose activities are overexpressed in the transformed state. In HNSCC tumor and cell lines, c-myc, TGF-α and EGF-R (epidermal growth factor receptor) are overexpressed, and RA inhibits the expression of TGF-α and EGF-R in several HNSCC cell lines [222-225]. Further, retinoids have been shown to down-regulate c-myc in other systems (Table V). SCC lesions overexpress squamous differentiation markers, such as TGase I, K1 and involucrin both *in vivo* and *in vitro* [for references, see 218]. The treatment of nonkeratinizing buccal epithelial cells with TPA results in increased involucrin and cross-linked envelopes which are characteristic of keratinized squamous epithelium [226]. Retinoids, which repress TPA-mediated gene expression [5], inhibit the expression of TGase I, K1 and involucrin in various HNSCC cell lines, thereby providing a mechanism of suppression of SCC differentiation and carcinoma growth [102, 218, 227].

Basal Cell Carcinoma (BCC)

BCC is the most widely occurring skin tumor. BCC occurs mostly in the head and neck regions of elderly patients with light skin. High doses of oral isotretinoin (13cis-RA; 1-4.6 mg/kg/day for 8 months) gave an approximately 40% clinical response, whereas, lower doses in the treatment of BCC were ineffective [228]. A clinical study in BCC with a combination treatment of oral 13cis-RA (0.2-0.4 mg/kg/day) and intralesional IFN-α (3×10^6 I.U., 3 times/week for 4 weeks) was conducted. The 4 week cycles were repeated after a one week interval in which only 13cis-RA was administered. An objective response was observed in 80% of the patients and 60% had complete response. At a dose of 0.2 mg/kg/day, the response rate was 75%, and it was 100% at 0.4 mg/kg/day [229]. Therefore, combinations of more potent receptor selective retinoids and IFN-α hold promise as an alternate to the traditional surgical approaches for the treatment of BCC.

Mycosis Fungoides (MF)

MF is a cutaneous T-cell lymphoma (CTCL), originating in the skin and progressing to the involvement of lymph nodes and visceral organs in later stages. MF has been shown to be responsive to 13cis-RA, etretinate and arotinoids [230-233]. 13cis-RA and etretinate treatments resulted in approximately 50% clinical response and 20-30% complete remissions [230, 233, 234]. Combination treatment of retinoids with IFN-α and PUVA therapy have been found to be more effective than single agent alone [235, 236]. In another study, although there was no significant difference between the 13cis-RA alone and PUVA+ 13cis-RA groups, remissions were obtained with a lower UVA dosage in the latter group [234]. Similarly, in the Mahrle and Theile (1987) study, there was no difference in the PUVA and etretinate + PUVA groups with respect to objective responses, but the latter group exhibited less toxicity. In a study with 10 MF patients, etretinate alone was not effective, whereas, significant clinical responses were observed in combination with PUVA [237].

Kaposi's Sarcoma (KS)

KS is the most common tumor associated with AIDS in homosexual or bisexual men. It develops predominantly in the skin, with occasional lymph node or visceral involvement [238]. Topical RA (tretinoin) and oral etretinate are clinically effective for the treatment of KS [152, 239]. Retinoids (RA, tazarotene and tazarotenic acid) have been shown to inhibit the proliferation of cultured KS cells [our unpublished results]. Retinoids inhibit KS cell growth by inhibiting the expression of IL-6, which is an autocrine growth factor for KS cells, but not the expression of other autocrine growth factors of KS cells, such as bFGF, VEGF and oncostatin-M.

Melanoma

This tumor is characterized by the proliferation of dendritic melanocytes and occurs predominantly in the skin but may also arise in the oral and nasal cavities, vulva, vagina, urethra, anus, the orbit and the meninges [240]. Most retinoids tested, including RA and arotinoid acid, had no significant inhibitory effect on the proliferation of melanoma cells in culture. However, the RARγ selective synthetic retinoids, (CD 437 and CD2325), exhibited a dose-dependent inhibition of proliferation of melanoma cells with IC_{50} (concentration required for 50% inhibition of cell proliferation) values ranging between 10^{-6} and 10^{-7}M. Unlike keratinocytes, which express only RARγ, melanoma cells express all three RAR types and the inactivity of agonists that activate all 3 RARs in melanoma cell proliferation is perplexing [241]. In the clinic, etretinate has given partial regression of cutaneous melanomas in individual cases but its overall effectiveness has not been documented [242, 243].

Cervical Dysplasia and Carcinoma

This tumor is the major cause of cancer related deaths among women worldwide and is the third most commonly occurring cancer in the US [244]. Human papillomaviruses (HPV) 16, 18,

31 and 33 have been found in more than 90% of the cervical tumor samples and among these HPV 16 is the virus type most commonly associated with the cancer. These associations suggest a role for HPV in the genesis of human cervical cancer [245, 246]. The virus produces two oncoproteins, E6 and E7, which are essential for the carcinogensis by inactivating growth suppressor proteins p53 and Rb, respectively, by direct protein: protein interaction [247-249]. The premalignant state of cervical carcinoma, cervical dysplasia has been reported to respond to retinoids. These clinical trials were triggered by an epidemiological study, which indicated greater risk of cervical dysplasia and carcinoma in women with a low dietary intake of β-carotene, vitamin A and ascorbic acid [250]. Another relevant epidemiological study suggested that serum retinol levels were lower in patients with various stages of cervical dysplasia than in women with normal squamous epithelium [251]. Retinyl acetate gel (up to 18 mg/day) when applied intravaginally for a week exhibited no significant toxicity in a phase I clinical trial, with maintenance or improvement in the dysplastic lesions [250]. In another clinical study, 18 women with cervical dysplasia were treated with RA, using an intravaginal collagen sponge. Although the vaginal toxic effects of RA were severe, 33% of the women showed improvement in their lesions. In another trial, RA was delivered by a cervical cap in 36 dysplastic women, and a complete response was noticed in 33% of the patients. In yet another clinical trial with RA, when drug was delivered every day for 4 days, followed by application for 2 days every 3 months for a period of 1 year, 50% of the patients achieved complete response. Overall, these clinical trials gave 33-50% complete responses in cervical dysplasia or mild cervical intraepithelial neoplasia (CIN I) [251-254]. In a phase III clinical study, designed to determine the efficacy of RA in reversing moderate CIN (CIN II) or severe CIN (CIN III), cervical caps containing 1 ml of RA (0.372%) or placebo were inserted daily for 4 days followed by placements for only 2 days at 3 month and 6 month periods. Complete regression was observed in 27% of placebo and 43% of RA treated patients in CIN II, whereas RA was not effective in CIN III patients [255]. In another trial involving 130 patients with documented signs of HPV infection, 13cis-RA in combination with IFN-β gave complete remission in 94.2% of the patients, whereas IFN-β alone gave a 88% response rate [256]. These clinical studies certainly suggest that retinoids are therapeutically effective in the treatment of cervical dysplasia (CIN I) or moderate neoplasia (CIN II). However, RA and 13cis-RA do not appear to be effective for advanced cervical carcinoma as single agents.

The therapeutic effects of retinoids in this disease are likely due to their direct action on viral gene expression and also because of their ability to influence the state of differentiation and proliferation of the HPV infected cells. RA inhibits the expression of HPV transforming oncogenes E6 and E7 at the RNA level in cervical cancer cells. This inhibition occurs at the level of transcription since the expression of a reporter construct containing HPV 16 or 18 regulatory regions is directly inhibited by RA in transfection experiments [84, 257]. Since HPV virion production is limited to the differentiating suprabasal cells, any agent which inhibits the differentiation state of epithelium, would interfere with the virus cycle [258]. Retinoids suppress the expression of differentiation markers K5, K16 and TGase I in HPV-16 immortalized cervical epithelial cell lines [259]. In a study comparing the sensitivity of normal human keratinocytes and HPV16 immortalized human keratinocytes, HPV16 immortalized keratinocytes were 10-100 fold more sensitive than normal keratinocytes to growth inhibition by RA [260]. These results indicate that RA may antagonize the effect of certain growth factors which are produced by the cell as a result of virus infection. Proliferation of HPV-16 immortalized cervical epithelial cells (ECE 16) is stimulated by epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1). Further, EGF enhances the growth stimulatory effects of IGF-1 by inhibiting the level of extracellular insulin-like growth factor binding protein-3 (IGFBP-3). RA has recently been shown to increase the expression of IGFBP-3 in the absence or presence of EGF. Thus, RA may inhibit the proliferation of HPV-infected cervical cells by increasing IGFBP-3 and thereby reducing the growth promoting activity of IGF-1 [261]. RA also inhibits the transformation of cells with HPV, presumably by changing the differentiation state of the cells [262, 263]. A combination of IFNα and RA was more effective than either treatment alone in inhibiting proliferation of HPV-infected human cervical carcinoma cells [264-266]. These results suggest that more selective retinoids with reduced toxicity should have considerable promise as single agents in the treatment and prevention of cervical dysplasia and in combination with agents such as IFN in the treatment of cervical cancer.

Lung Cancer

Since retinoids have profound effects on the proliferation and differentiation of epithelial cells and they have demonstrated therapeutic activity in other epithelial premalignancies and malignancies (e.g., SCC, HNSCC, BCC and cervical dysplasia), they are an attractive therapeutic modality for the treatment of premalignant and malignant lesions of the lung. Vitamin A deficiency has been shown to result in hyperplasia and squamous metaplasia of tracheobronchial epithelial cells because of the inability of the cells to differentiate along the normal mucosecretory pathway and supplementation of dietary vitamin A can reverse squamous metaplasia [267-270]. The absence of tracheobronchial mucosecretory differentiation or incomplete differentiation is observed both in lung carcinogenesis and vitamin A deficiency [269, 270]. Further, retinoid and vitamin A-dependent inhibition of chemically-induced tracheobronchial hyperplasia, metaplasia and squamous tumor has been reported [271, 272]. As discussed previously, the RARβ gene expression is decreased in most small cell lung carcinoma cell lines and even in the primary lung tumor tissues [191,193]. Taken together, these observations suggest a perturbance in the retinoid signal transduction pathway during lung cancer development. RA, apart from inhibiting the malignant phenotype and tumor cell growth, suppresses the colonogenic potential and, chemotactic and chemoinvasive capacity of a highly metastatic lung carcinoma cell line C87. The inhibition of the malignant and metastatic phenotype is accompanied by a marked reduction in the expression of β4 integrin, an adhesion molecule, which is abundant in lung and colon carcinoma and is associated with the metastatic ability of the tumor [91, 273-276].

In the clinic, 13cis-RA and etretinate have been used in patients with bronchial squamous metaplasia but the results were not encouraging [277-280]. Similarly, 13cis-RA alone or in

combination with IFN-α were not therapeutically effective (5-10% clinical response) in non-small cell lung cancer and in squamous cell lung cancer [281-282]. It is very possible that 13cis-RA and etretinate are not potent enough for the treatment of lung premalignancy. Although the clinical results with the use of retinoids in lung cancer have been disappointing so far, the sound theoretical basis for their potential therapeutic value in this cancer warrants further studies with newer receptor selective retinoids. In this regard, RARβ selective retinoids are a particularly intriguing possibility.

Breast Cancer

Breast cancer is the most commonly occurring tumor amongst women in the western world and the incidence of this cancer is on the rise. Inhibition of the growth of ER-positive breast cancer cell lines by retinoids has been discussed earlier. In a study involving induction of experimental mammary cancer with N-nitro-N-methylurea in Sprague-Dawley rats, oral 9cis-RA (120 or 60 mg/kg of diet) was effective in reducing tumor incidence, average number of tumors per rat and extending tumor latency. An additive effect was seen in combination with tamoxifen. Surprisingly, all-trans RA was not as effective as 9cis-RA when used alone or in combination with tamoxifen in this model system, even though both retinoids were equally potent in inhibiting the proliferation of breast cancer cells and in inducing the expression of adhesion molecule E-cadherin *in vitro* in the SK-BR3 breast cancer cell line [283]. The additive or synergistic effects of IFN-γ and tamoxifen and the excellent antiproliferative effects of retinoids *in vitro* in breast cancer cell as well as *in vivo* (without and with tamoxifen) in an experimental breast cancer model, suggest that retinoids have tremendous potential as a therapeutic modality particularly in combination with other cytotoxic or cytostatic agents for the treatment of breast cancer.

In a recent phase II clinical trial, 49 metastatic breast cancer patients were treated with tamoxifen (30 mg/days), IFN-β (1-3 x 10^6 IU/m^2, subcutaneously, thrice a week) and retinyl palmitate (15,000-50,000 IU, orally, twice per day). Complete response was observed in 12 patients (24%), partial response in 15 patients (31%), stable disease in 10 (20%) and disease progressed in 12 patients (25%). The median response rate was 31.4 months. The study showed that the long-term administration of the combination of these drugs is feasible with moderate toxicity [284]. Retinoids can also be effective in drug-resistant breast cancer, since an arotinoid Ro 40-8757, in contrast with RA, inhibited the proliferation of a drug resistant breast cancer line MCF-7 mdr 1. The mechanism of action of this arotinoid is unknown, since it does not bind to any of the known RARs or RXRs [285]. The amplification of c-myc protooncogene in breast cancers, the association of elevated c-myc with carcinogenesis in experimental animals and with invasiveness in human breast cancers, suggest that the abnormal regulation of c-myc might play a part in the pathogenesis of breast cancer [286-288]. c-myc expression is down-regulated by hydroxy-tamoxifen, methoxyprogesterone acetate, RA, dexamethasone and calcitriol, the anti-proliferative hormonal agents which inhibit the growth of breast cancer cells *in vitro* [80,289-291]. Therefore, apart from IFN-β and tamoxifen, retinoids might prove to be useful in combination with 1,25-dihydroxyvitamin D_3 and /or dexamethasone in this tumor.

Ovarian Cancer Cells

There are only a few reports on the growth inhibitory effects of RA in ovarian cancer cells [292-294]. In ovarian cancer cell lines which are most sensitive to RA-inhibition (NIHOVCAR and OVCCR1), the response is associated with morphological changes characteristic of cell differentiation, while the RA-resistant cell line IGROV1 does not exhibit a differentiated phenotype [294]. The association of more differentiated ovarian tumors with higher survival rates suggests that the differentiation inducing capabilities of retinoids may be harnessed for the treatment of ovarian cancer. An RARβ receptor expression-deficient ovarian cancer call line, HOC-7, which can be differentiated by dimethylformamide, TGF-β and DMSO, was un-responsive to RA induced differentiation [295,296], but exhibited RA-induced apoptosis when cultured under low serum condition. The apoptosis was accompanied by RA-stimulation of c-myc expression, unlike in other various cancer system where RA-inhibits c-myc expression [296]. Therefore, RA can achieve the endpoint of anti-proliferative effects in these tumor cells both by differentiation and apoptotic pathways. Ovarian cancer cells express RARα and RARγ as the major isotypes, and RARβ was below the detection limits of this study. RARγ was not expressed in the RA-resistant ovarian cancer cell line IGROV1 [296]. Therefore, RARα and/or RARγ-selective synthetic retinoids might have the most therapeutic potential in this disease.

Acute Myeloid Leukaemia (AML)

AML is a group of acute cancers of bone marrow which can be classified into seven different types of myeloid leukaemias (M1 through M7), depending upon the stage of arrest of the differentiation program of the myeloid cells. APL, the most retinoid-responsive tumor, is an M3 AML because of a differentiation block from promyelocytes to granulocytes [297, 298]. The most widely studied leukaemic cell line, HL-60, is from a myelobalstic leukaemia (M1) patient [299, 300]. Treatment of HL-60 cells with RA or 13cis-RA terminally differentiates them on a granulocyte pathway [301, 302]. The rationale that RA is a potent differentiation agent of HL-60 cells in culture, led to a study of the effect of RA on primary leukaemic cells [303]. Of 21 patients with primary myeloid leukaemia, only the cells of APL patients (n=2) responded to RA-mediated differentiation. It was later demonstrated that the cells of all APL patients differentiate in response to nanomolar concentrations of RA [304]. In a clinical trial with 21 children with AML, who underwent remission following cytostatic agents, 50,000 IU retinol/m^2 was administered daily for up to 3 years. Fourteen of the patients remained in remission even after 11 years of the start of the therapy. This trial established the use of retinol in addition to cytostatic agents in AML children as a much better regimen than the use of cytostatic agent alone [305, 306]. In a clinical trial of APL patients (n=26), complete clinical remission was obtained with RA in all the patients [307]. Similar astonishing results were also observed in French and American clinical trials [308, 309]. Since myeloid cells contain RARα as the major isotype, the use of RARα-selective retinoids might provide better therapeutic indices in APL and other leukaemias.

Prostate Cancer

Since retinoids successfully inhibit the proliferation of malignant epithelial cells and vitamin A deficiency results in male reproductive system disorders [310, 311], various laboratories have studied the effect of retinoids on the growth of prostatic cells. Retinoids inhibits the proliferation of both normal and cancerous prostate cells *in vitro* and *in vivo* [312-314]. Aberrant activation of the androgen receptor (AR) by androgens has been implicated in the progression of prostatic carcinoma [315-316]. In addition, growth factors IGF-1, KGF (Keratinocyte growth factor) and EGF induce the androgen signal transduction pathway [316]. Retinoids may function in prostatic cancer cells by antagonizing the growth promoting actions of AR, since androgen-induced expression of prostate-specific antigen (PSA) and human glandular kalikrein-1 (hKLK2) is significantly repressed by RA in a dose-and time-dependent manner [314].

Neuroblastoma

All-trans RA has been consistently shown to inhibit the proliferation of neuroblastoma cells and induce their differentiation *in vitro* [101, 317, 318]. Childhood neuroblastoma has the highest rate of spontaneous regression through differentiation, and RA-mediated differentiation of neuroblastoma cells is characterized by cell cycle arrest and neurite outgrowth [79, 318]. Higher levels of RARγ have been shown to correlate with the RA-mediated growth inhibition of human neuroblastoma cells [318]. RA inhibits the expression of proto-oncogene N-myc and IGF binding protein (IGFBP)-2 and 4 in human neuoblastoma cells [79, 101] and these events may play a role in the retinoid-dependent differentiation of neuroblastoma cells. 9cis-RA is a better inducer of differentation, RARβ induction and CRABPII induction in SM-S4-5Y neuroblastoma cells, as compared to RA and 13cis-RA [319]. In a clinical trial, oral 13 cis-RA was not significantly effective in children with metastatic neuroblastoma. Since the subjects considered in this study were unresponsive to chemotherapy, the possibility that these patients were also unusually resistant to retinoid therapy cannot be excluded. Based upon the *in vitro* data with neuroblastoma cells, it appears that better newer generation retinoids, such as 9cis-RA analogs and RARγ selective retinoids, either alone or in combination with other chemotherapy regimens, may yield significant clinical responses in this tumor. RA also inhibits the growth and induces differentiation of glioblastoma-astrocytoma cells in culture [320].

Multiple Myeloma (MM)

RA alone or in combination with IFN or dexamethasone inhibits the growth of myeloma cells *in vitro* [321-325]. 13cis-RA is a better growth inhibitor of myeloma cells than all-trans-RA [324]. IL-6 is a major autocrine and/or paracrine growth factor for myeloma cells [326-329]. Retinoids and glucocorticoids both appear to inhibit the proliferation of myeloma cells by inhibiting the expression of IL-6 (by glucocorticoids) and IL-6 receptor (by retinoids) [90, 321, 330]. Since RA inhibits IL-6 expression in KS cells, it may also interfere with the IL-6 signal transduction pathway in myeloma cells by directly repressing expression of the IL-6 gene. However, treatment of multiple myeloma patients (n=6) with RA was ineffective and it led to hypercalcaemia and increased IL-6 levels [331]. Since induction of gene expression by RARs is achieved by virtue of their activation functions (AF-1 and AF-2), the use of retinoids which are inert in their transactivation functions, while still active in their inhibitory functions, may prove therapeutically effective for the treatment of MM.

Future of Retinoids in Cancer Therapy

The therapeutic efficacy can be increased and associated toxic side effects reduced by the use of receptor selective synthetic retinoids. The selection of a synthetic retinoid will depend upon the cancer cell type, the complement of retinoid receptors present in a particular cancer, and the association of a receptor subtype with the therapeutic and/or toxic effects of retinoid therapy.

Since AP1 is involved in cell growth, the use of pure anti-AP1 and transactivation inert retinoids in the treatment of hyperproliferative and inflammatory diseases may be very beneficial.

Like RAR-AP1 interactions, the fundamental pathways of RAR action on cell growth and differentiation appear to intersect with signal transduction pathways of ER and AR. Therefore, retinoids with potent ER- and AR-antagonism properties (but with reduced retinoid-dependent transactivation potential) could be therapeutically effective for the treatment of ER-dependent breast cancer, uterine hyperplasia and prostate cancer.

Retinoids in combination with other biological response modifiers, like, IFN, dexamethasone, vitamin D_3 as well as cytotoxic/cytostatic agents may offer additive or synergistic effects, as indicated by various *in vivo* and *in vitro* studies. These combinations may in addition have reduced toxicities in comparison to the use of individual agents alone.

The use of new preparations of retinoids (e.g., liposome encapsulated retinoids) for better drug delivery and improved pharmacokinetic properties will also be helpful.

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